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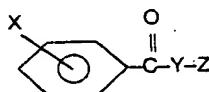
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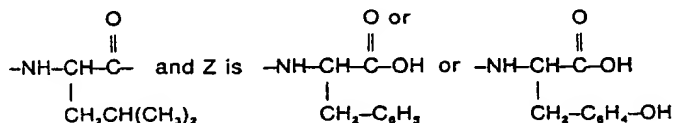
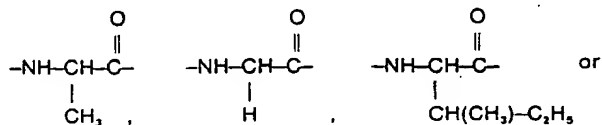
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54 Dipeptide derivatives and their use in determining the activity of carboxypeptidase A.

57 Dipeptide derivatives useful as substrates for colourimetrically or spectrophotometrically measuring the activity of carboxypeptidase A have the following general formula



wherein X is OH in the ortho or para position, or NH<sub>2</sub> or CH<sub>3</sub>O in the para position, Y is



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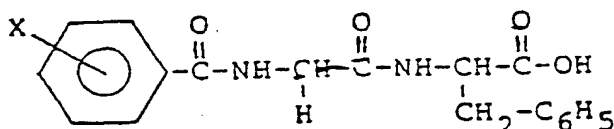
EP 0 150 227 A1

1 "DIPEPTIDE DERIVATIVES AND THEIR USE IN DETERMINING THE  
ACTIVITY OF CARBOXYPEPTIDASE A"

This invention relates to novel dipeptide derivatives and to their use as substrates for measuring  
 5 the activity of carboxypeptidase A (hereinafter referred to as CP-A for short).

CP-A is a protein-decomposing enzyme which is found in the pancreas and in blood serum. The activity of CP-A depends on the disease which is present and its  
 10 extent. Accordingly, by measuring the activity of the CP-A, the extent at which a disease has spread can be measured.

Our European Patent Application EP-A1-0083857 discloses two derivatives of hippuryl-L-phenylalanine  
 15 having the following general formula



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wherein X is OH or CH<sub>3</sub>O. These dipeptides are useful as substrates for measuring the activity of carboxypeptidase A. The activity measuring method which has been  
 25 described comprises the steps of:

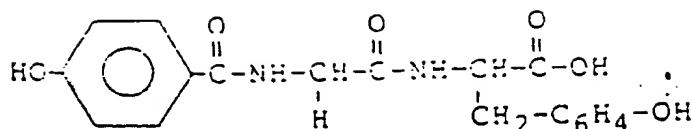
(1) bringing into contact with each other in a liquid medium containing CP-A, (i) such a hippuryl-L-phenylalanine derivative as substrate, (ii) hippuricase, (iii) 4-amino-antipyrine and (iv) an oxidising agent  
 30 capable as such of oxidising X-benzoic acid produced in situ to yield a substance which reacts with 4-aminoantipyrine present to yield a quinonimine dye;

(2) colourimetrically measuring the concentration of the quinonimine dye and

35 (3) calculating the activity of said enzyme from the concentration of the quinonimine dye.

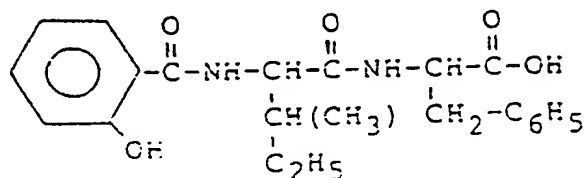
It is an object of this invention to provide

1 2.



5 (p-hydroxybenzoylglycyl-L-tyrosine)

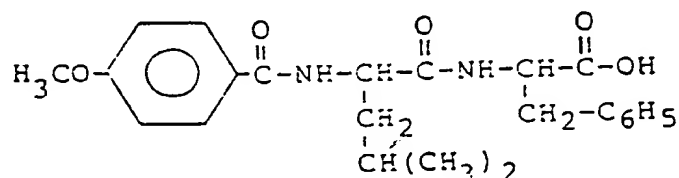
3.



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(o-hydroxybenzoyl-L-isoleucyl-L-phenylalanine)

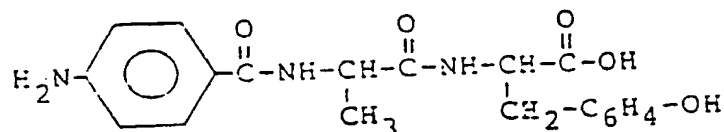
4.



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(p-methoxybenzoyl-L-leucyl-L-phenylalanine)

5.



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(p-aminobenzoyl-L-alanyl-L-tyrosine)

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According to a second aspect of this invention there are provided methods for determining colourimetrically or spectrophotometrically the activity of CP-A. Thus a first method comprises the steps of

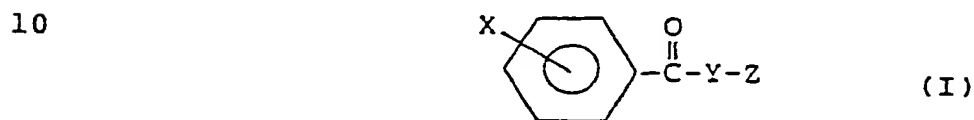
(1) bringing into contact with each other in a liquid medium containing the enzyme carboxypeptidase A, (i), as substrate, a dipeptide derivative of the invention, (ii) hippuricase, (iii) 4-aminoantipyrine and (iv) an oxidising agent capable as such of oxidising X-benzoic acid, obtainable from the dipeptide produced in situ to yield a substance which reacts with 4-aminoantipyrine present to yield a quinonimine dye;

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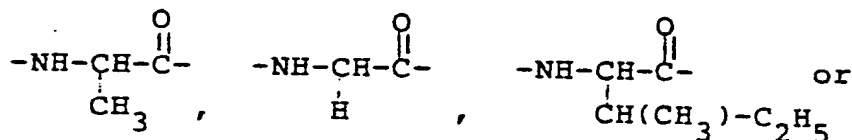
(2) colourimetrically measuring the concentration

- 1 alternative substrates and/or alternative activity  
measuring procedures for determining the activity of  
carboxypeptidase-A, which is/are able to offer like or  
superior effectiveness in the determination of the  
5 activity of carboxypeptidase-A.

According to one aspect of this invention, there  
are provided dipeptide derivatives which possess the  
following general formula:



wherein X is OH in the ortho or para position, or NH<sub>2</sub> or  
15 CH<sub>3</sub>O in the para position, Y is

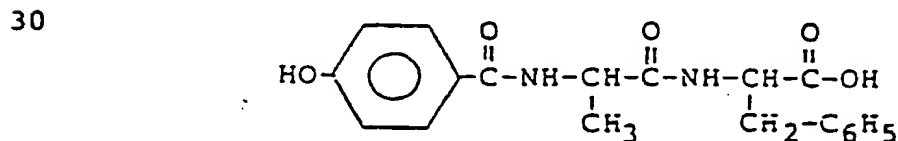


20 
$$\begin{array}{c} \text{O} \\ \parallel \\ \text{-NH-CH-C-} \\ | \\ \text{CH}_2\text{CH(CH}_3\text{)}_2 \end{array} \quad \text{and Z is} \quad \begin{array}{c} \text{O} \\ \parallel \\ \text{-NH-CH-C-OH} \\ | \\ \text{CH}_2\text{-C}_6\text{H}_5 \end{array} \quad \text{or} \quad \begin{array}{c} \text{O} \\ \parallel \\ \text{-NH-CH-C-OH} \\ | \\ \text{CH}_2\text{-C}_6\text{H}_4\text{-OH} \end{array}$$
  
with the proviso that when X is OH or CH<sub>3</sub>O, Y is not

25 
$$\begin{array}{c} \text{O} \\ \parallel \\ \text{-NH-CH-C-} \\ | \\ \text{H} \end{array} \quad \text{with Z being} \quad \begin{array}{c} \text{O} \\ \parallel \\ \text{-NH-CH-C-OH} \\ | \\ \text{CH}_2\text{-C}_6\text{H}_5 \end{array}$$

Specific examples of dipeptide derivatives  
embodying this invention are the following compounds:

1.



(p-hydroxybenzoyl-L-alanyl-L-phenylalanine)

35

1 of the quinonimine dye and

(3) calculating the activity of said enzyme from the concentration of the quinonimine dye.

A second method comprises the steps of

5 (1) bringing into contact with each other in a liquid medium containing the enzyme carboxypeptidase A, (i), as substrate, a dipeptide derivative of the invention (ii) hippuricase, (iii) NADPH or NADH and (iv) a benzoate hydroxylase having an identity corresponding  
10 to the meaning and position of X in the formula of the dipeptide derivative;

(2) spectrophotometrically measuring the decrease in Absorbance by NADPH or NADH at about 340 nm of light;

(3) calculating the activity of said enzyme from  
15 the decrease in absorbance by NADPH or NADH.

By the reference to the benzoate hydroxylase having an identity corresponding to the meaning and position of X in the formula of the dipeptide derivative, it is meant for example, that if X is p-OH, then a  
20 p-hydroxybenzoate hydroxylase will be used.

A third method comprises the steps of:

(1) bringing into contact with each other in a liquid medium containing the enzyme carboxypeptidase A, (i), as substrate, a dipeptide derivative of the  
25 invention wherein X is essentially  $\text{-NH}_2$  and (ii) hippuricase;

(2) adding  $\text{NaNO}_2$  to the reaction mixture to produce a diazonium compound;

(3) adding a coupling agent to produce an azo  
30 dye;

(4) colourimetrically measuring the concentration of the azo dye; and

(5) calculating the activity of said enzyme from the concentration of the azo dye.

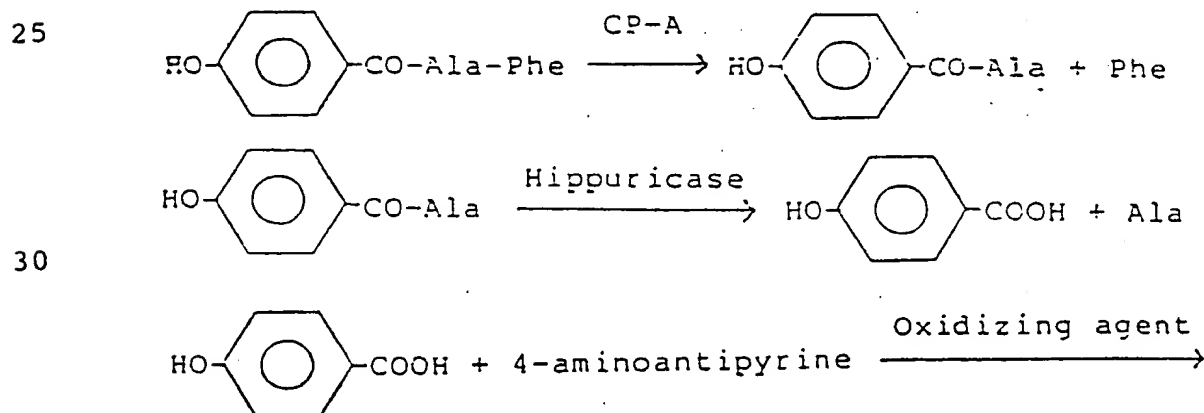
35 In a third aspect, the invention provides a diagnostic composition for use in determining the activity of carboxypeptidase A, which, in its simplest

1 form, comprises a dipeptide compound of the invention and hippuricase.

When the composition is to be used in the first diagnostic method as aforesaid, it may additionally contain 4-aminoantipyrine and an oxidising agent capable as such of oxidising X-benzoic acid, in situ in the composition to yield a substance which reacts with 4-aminoantipyrine to yield a quinonimine dye. When the composition is to be used in the second diagnostic method it may additionally contain NADPH or NADH and a benzoate hydroxylase having an identity corresponding to the meaning and position of X in the dipeptide compound present.

The three diagnostic methods will now be described more fully with reference to specific reaction schemes. In the following, "Ala", "Phe", "Gly" and "Tyr" are used as abbreviations for "alanyl or alanine", "phenylalanyl or phenylalanine", "glycyl or glycine" and "tyrosyl or tyrosine", respectively, and further "NADH" means "reduced nicotinamide adenine dinucleotide" and "NADPH" means "reduced nicotinamide adenine dinucleotide phosphate".

1.



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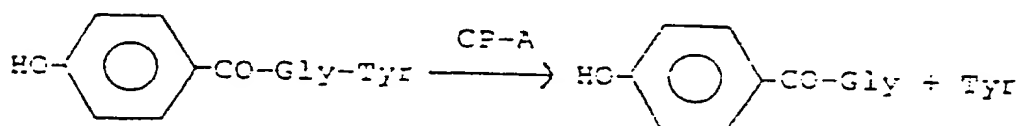
Quinonimine dye

The concentration of the quinonimine dye produced

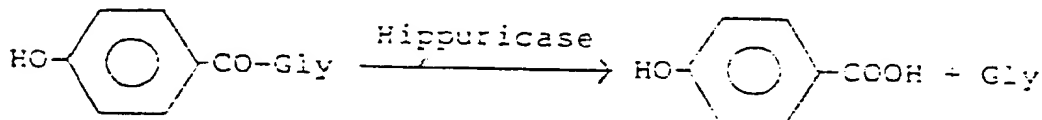
1 may be colourimetrically measured, and the activity of  
 CP-A is then calculated. As in EP1-A-0083857, the  
 oxidising agent used may be, for example, periodic acid  
 or a periodate perchloric acid or a perchlorate,  
 5 potassium ferricyanide, ammonium ceric nitrate,  
 chloramine T, chloramine B or chromium trioxide.  
 Preferred procedures using the colourimetric method will  
 be illustrated in the following Examples 6 and 7.

2.

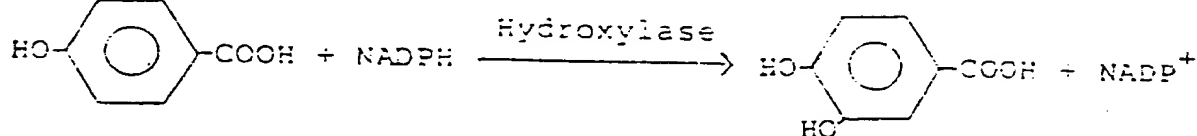
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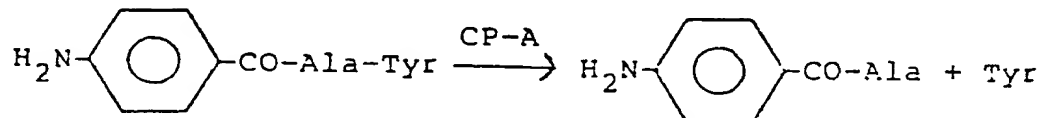


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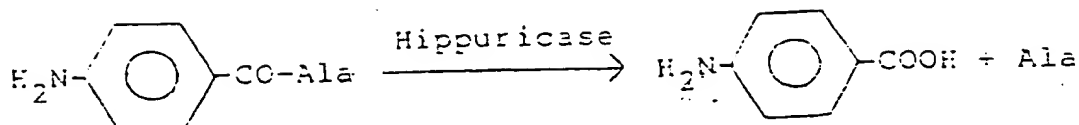
The decrease of the absorbance by NADPH is  
 spectrophotometrically measured, at an appropriate  
 25 absorption band, preferably at 340 nm of light, during  
 the course of the reaction using a reaction rate  
 analyser, and the activity of CP-A is calculated.  
 Preferred procedures using this spectrophotometric  
 technique will be illustrated in the following Examples 8  
 30 and 9. In Example 10, NADH is used instead of NADPH in  
 an otherwise like procedure.

3.

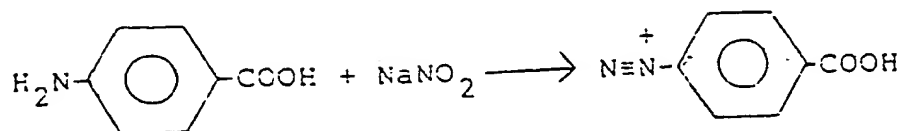


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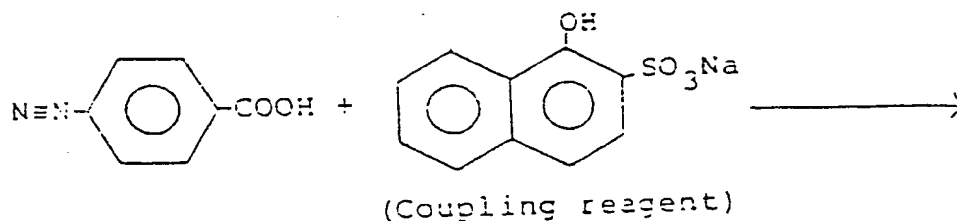
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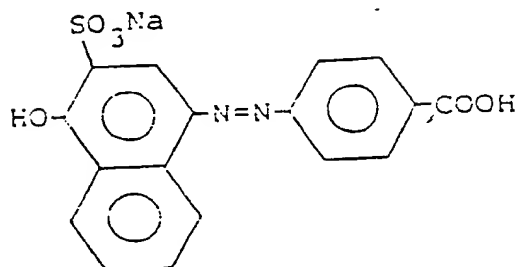
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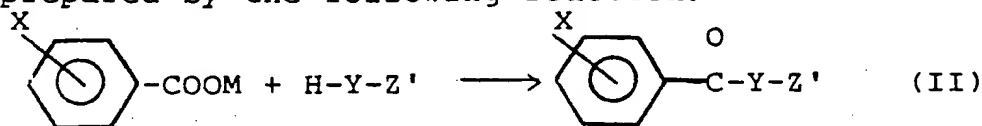
(Azo dye)

The concentration of the azo dye produced is colourimetrically measured, and the activity of CP-A is calculated. A preferred procedure using this colourimetric method will be illustrated in the following Example 11.

25

The dipeptide derivatives of this invention can be prepared by the following reaction:

30



35

wherein X and Y have the aforesaid meanings wherein Z' is an esterification product of Z. This reaction is usually carried out in an organic solvent such as dicyclohexylcarbodiimide, and the compound of general formula (I) can be obtained by hydrolysis of the compound



1 having the general formula (II).

The following Examples illustrate this invention, Examples 1 to 5, illustrating the preparation of dipeptide derivatives of the present invention and the remaining Examples illustrating the use of such compounds in the determination of CP-A.

EXAMPLE 1

Synthesis of p-hydroxybenzoyl-L-alanyl-L-phenylalanine:

10 0.1 g (0.1 mol) of triethylamine was added to a suspension of 23.0 g (0.1 mol) of L-phenylalanine ethyl ester.HCl in 500 ml of dichloromethane. This mixture was placed in an ice bath and 22.3 g (0.1 mol) of carbobenzoxy-L-alanine, and then 19.2 g (0.1 mol) of  
15 l-ethyl-3(3-dimethylaminopropyl) carbodiimide.HCl were added thereto. The mixture obtained was stirred for three hours and then were washed three times with a 0.1% aqueous solution of  $\text{Na}_2\text{CO}_3$ , three times with a 0.1 N HCl solution and then three times with distilled water.  
20 Thereafter, the mixture was dried over anhydrous sodium sulphate, and dichloromethane was removed from the mixture under reduced pressure to leave carbobenzoxy-L-alanyl-L-phenylalanine ethyl ester. This ester was dissolved in 260 ml of 25% HBr in glacial  
25 acetic acid, and the solution obtained was stirred for one hour. 5 Litre of absolute ethyl ether were added to this solution to produce a precipitate. The precipitate was filtered and washed with diethyl ether. L-alanyl-L-phenylalanine ethyl ester.HBr was thereby  
30 obtained.

17.3 g (0.05 mol) of L-alanyl-L-phenylalanine ethyl ester.HBr were suspended in 250 ml of dichloromethane, and to this suspension were added 5.1 g (0.05 mol) of triethylamine and 9 g (0.05 mol) of  
35 p-acetoxybenzoic acid. The mixture obtained was placed in an ice-bath and then 9.6 g (0.05 mol) of l-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl were added. The

1 mixture then obtained was stirred for three hours, and  
then was washed three times with a 0.1% aqueous solution  
of  $\text{Na}_2\text{CO}_3$ , three times with a 0.1 N HCl solution and  
three times with distilled water, before being dried over  
5 anhydrous sodium sulphate. Dichloromethane was removed  
from the dried mixture under reduced pressure to yield  
p-acetoxycarbonyl-L-alanyl-L-phenylalanine ethyl ester.

8.5 g (0.02 mol) of the ester thus obtained were  
dissolved in 50 ml of methanol, and to this solution,  
10 placed in an ice bath, were added 70 ml of a 1 N NaOH  
aqueous solution. The solution obtained was stirred for  
two hours at room temperature. After the pH of the  
solution had been adjusted to 7.0 with a 1 N HCl  
solution, the solvent was distilled off from the solution  
15 under reduced pressure. The residue of p-hydroxy-  
benzoyl-L-alanyl-L-phenylalanine obtained was purified by  
absorption chromatography. 6.4 g of residue m.p.  
211°C-211.6°C (decomposed) were obtained. Its infrared  
absorption spectrum is shown in Figure 1 of the  
20 accompanying drawings.

#### EXAMPLE 2

Synthesis of p-hydroxybenzoylglycyl-L-tyrosine:

21 g (0.1 mol) of carbobenzoxyglycine were  
dissolved in 500 ml of dichloromethane, and 24.5 g (0.01  
25 mol) of L-tyrosine ethyl ester were added to this  
solution. The mixture obtained was placed on an  
ice-bath and 19.2 g (0.1 mol) of 1-ethyl-3(3-dimethyl-  
aminopropyl)carbodiimide.HCl were added. The reaction  
solution obtained was stirred overnight.

30 The reaction mixture was washed three times with a  
0.1% aqueous solution of  $\text{Na}_2\text{CO}_3$  and three times with  
distilled water, and then was dried over anhydrous sodium  
sulphate. Dichloromethane was removed from the reaction  
mixture under reduced pressure to leave behind  
35 carbobenzoxyglycyl-L-tyrosine ethyl ester.

250 ml of 25% HBr in glacial acetic acid were  
added to carbobenzoxyglycyl-L-tyrosine ethyl ester

1 obtained above, and, after the mixture obtained had been  
stirred for 30 minutes at room temperature, 5 litres of  
absolute ethyl ether were added to the mixture to produce  
a precipitate. The precipitate of glycyl-L-tyrosine  
5 ethyl ester.HBr obtained was washed with diethyl ether.

17.4 g (0.05 mol) of glycyl-L-tyrosine ethyl  
ester.HBr, 9 g (0.05 mol) of p-acetoxybenzoic acid and  
5.1 g (0.05 mol) of triethylamine were dissolved in 250  
ml of dichloromethane, and after the solution had been  
10 cooled to 0°C, 9.6 g (0.05 mol) of 1-ethyl-3(3-  
dimethylamino-propyl)carbodiimide.HCl were added to the  
solution, and the solution was stirred overnight. The  
reaction mixture obtained was washed with a 0.1% aqueous  
solution of Na<sub>2</sub>CO<sub>3</sub> and with a 0.1 N HCl solution, and was  
15 dried over anhydrous sodium sulphate. Dichloromethane  
was removed from the mixture under reduced pressure and  
p-acetoxybenzoylglycyl-L-tyrosine ethyl ester was  
recovered.

8.6 g (0.02 mol) of p-acetoxybenzoylglycyl-L-  
20 tyrosine ethyl ester obtained above were dissolved in 50  
ml of methanol, and to this solution were added 70 ml of  
a 1 N NaOH aqueous solution. The solution obtained was  
stirred for one hour at room temperature. The pH of the  
solution was adjusted to 7.0 with a 1 N HCl solution, and  
25 the solvent was distilled off from the solution under  
reduced pressure. The residue was dissolved in  
methanol, and the insoluble matter was filtered off. To  
the filtrate was added diethyl ether to produce a  
precipitate. This precipitate was filtered off and  
30 p-hydroxybenzoylglycyl-L-tyrosine in an amount of 5 g.  
m.p. 184°C-210°C (decomposed) was obtained. Its  
infrared absorption spectrum is shown in Figure 2 of the  
accompanying drawings.

### EXAMPLE 3

35 Synthesis of o-hydroxybenzoyl-L-isoleucyl-  
L-phenylalanine:

4.6 g of o-hydroxybenzoyl-L-isoleucyl-

- 1 L-phenylalanine were obtained by repeating a procedure analogous to that set out in Example 2, using 26.5 g of carbobenzoxyisoleucine instead of 21 g of carbobenzoxyglycine, 20 g of L-phenylalanine ethyl ester.HCl instead
- 5 of 24.5 g of L-tyrosine ethyl ester.HCl, and 9 g of o-acetoxybenzoic acid instead of 9 g of p-acetoxybenzoic acid.

#### EXAMPLE 4

10 Synthesis of p-methoxybenzoyl-L-leucyl-L-phenylalanine:

The procedure of Example 2 was repeated, but using 26.5 g of carbobenzoxy-L-leucine instead of 21 g of carbobenzoxyglycine and 7.6 g of p-methoxybenzoic acid instead of 9 g of p-acetoxybenzoic acid, to obtain 3.6 g

15 of p-methoxybenzoyl-L-leucyl-L-phenylalanine.

#### EXAMPLE 5

Synthesis of p-aminobenzoyl-L-alanyl-L-tyrosine:

4.4 g of p-aminobenzoyl-L-alanyl-L-tyrosine were obtained by repeating the procedure of Example 2, but

20 using 22.3 g of carbobenzoxy-L-alanine instead of 21 g of carbobenzoxyglycine and 8.9 g of p-acetamidobenzoic acid instead of 9 g of p-acetoxybenzoic acid.

#### EXAMPLE 6

0.1 ml of serum containing carboxypeptidase A was

25 added to 0.5 ml of a test reagent solution of pH 7.8 containing 10 mM p-hydroxybenzoyl-L-alanyl-L-phenylalanine, 0.1 M sodium chloride, 3000 U/l hippuricase, 2.5 mM 4-aminoantipyrine and 100 mM N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid.

30 The mixture was incubated at 37°C for 20 minutes. 1.5 ml of a reaction termination solution containing 6.5 mM sodium periodate was added to this reaction mixture. The mixture obtained was incubated at 37°C for five minutes, so that quinonimine dye was formed. The

35 absorbance of the reaction mixture was measured at a light wavelength of 505 nm. This absorbance is referred to as Absorbance A.

1 Separately, for a blank (comparison) test, in the  
 procedure as mentioned above, 1.5 ml of a reaction  
 termination solution containing 6.5 mM sodium periodide  
 was added to the test reagent solution before 0.1 ml of  
 5 serum containing carboxypeptidase A was added to the test  
 reagent solution. The absorbance of the blank test  
 reagent was then measured in the same manner as described  
 above, at a light wavelength of 505 nm. This absorbance  
 is referred to as Absorbance B.

10 The activity value (mU/ml) of carboxypeptidase A  
 can be calculated by the following equation:

$$\text{mU/ml} = \frac{A - B}{\text{Molecular extinction coefficient } (\xi)} \times \frac{1}{\text{Reaction time (5 min.)}} \times \frac{1}{\text{Light path length (cm)}} \times$$

15  $\frac{2.1}{0.1} \times 10^6$

where  $\xi = 12000$  (Molecular extinction coefficient of quinoxaline dye)

20 By repeating the above-described test 20 times,  
 using the same samples, the following results were  
 obtained:

Activity value (mU/ml) = 5.1 (nmol/ml/min)

25 Coefficient of variation (CV) = 3.4%.

#### EXAMPLE 7

The activity of CP-A was determined by repeating  
 the procedure of Example 6, except that (a) p-hydroxy-  
 benzoylglycyl-L-tyrosine or (b) o-hydroxybenzoyl-L-  
 30 isoleucyl-L-phenylalanine was used instead of  
 p-hydroxybenzoyl-L-alanyl-L-phenylalanine.

The following results were obtained:

(a) Activity value (mU/ml) = 2.4 (nmol/ml/min)

(b) " " = 1.2 "

35 (a) Coefficient of variation (CV) = 4.5%

(b) " " = 6.0%.

EXAMPLE 8

0.05 mol of serum (the same as used in Example 6) was added to 0.5 ml of a test reagent solution (pH 8.0) containing 10 mM p-hydroxybenzoylglycyl-L-tyrosine, 3000 U/l hippuricase, 0.2 mM NADPH, 10000 U/l p-hydroxybenzoate hydroxylase and 100 mM boric acid.

The decrease in the absorbance by the solution was measured at 340 nm of light at a temperature of 37°C during the course of the reaction. There was used for the measurement a reaction rate analyser in which the reaction time was five minutes. The activity value (mU/ml) of CP-A was automatically printed out in the analyser.

The calculation of activity was performed according to the following formula:

$$\text{mU/ml} = \frac{\text{Decrease in absorbance at 340 nm}}{\text{Reaction time (5 min)}} \times \frac{1}{\text{Molecular extinction coefficient } (\epsilon)} \times \frac{1}{\text{Light-path length (cm)}} \times \frac{0.55}{0.05} \times 10^6$$

wherein  $\epsilon = 6.22 \times 10^3$  (Molecular extinction coefficient of NADPH)

The results of the measurements, repeated 20 times, were as follows:

Activity value (mU/ml) = 6.2 (nmol/ml/min)  
Coefficient of variation (CV) = 5.2%.

EXAMPLE 9

The activity of CP-A was determined by repeating the procedure shown in Example 8 except that o-hydroxybenzoyl-L-isoleucyl-L-phenylalanine was used instead of p-hydroxybenzoylglycyl-L-tyrosine, salicylate hydroxylase was used instead of p-hydroxybenzoate hydroxylase, and nicotinamide adenine dinucleotide in reduced form (NADH) was used instead of NADPH.

1       The following results were obtained:  
Activity value (mU/ml) = 1.1 (nmol/ml/min)  
Coefficient of variation (CV) = 7.0%

EXAMPLE 10

5       The activity of CP-A was determined by repeating  
the procedure shown in Example 8 with a mixture prepared  
by adding 0.05 ml of serum (the same as used in Example  
6) to 0.5 ml of a test reagent solution (pH 8.0)  
containing 30 mM p-methoxybenzoyl-L-leucyl-L-  
10 phenylalanine, 1000 U/l hippuricase, 0.2 mM NADH, 20000  
U/l p-methoxybenzoate hydroxylase and 100 mM phosphoric  
acid.

      The following results were obtained:  
Activity value (mU/ml) = 2.5 (nmol/ml/min)  
15       Coefficient of variation (CV) = 4.7%.

EXAMPLE 11

      0.05 ml of serum (the same as used in Example 6)  
containing CP-A was added to 0.5 ml of a test reagent  
solution (pH 8.0) containing 20 mM p-aminobenzoyl-L-  
20 alanyl-L-tyrosine, 0.5 M NaCl, 3000 U/l hippuricase and  
100 mM boric acid. After this mixture had been allowed  
to stand at 37°C for 20 minutes, 0.5 ml of a solution  
containing 2 mM NaNO<sub>2</sub> and 1 mM NaOH was added to the  
mixture, and the mixture was allowed to stand at room  
25 temperature for 10 minutes. 0.5 ml of a reagent  
solution containing 1 mM potassium 1-naphthol-2-sulphate,  
50 mM boric acid and 200 mM NaOH was added to the  
mixture. The absorbance by the solution was measured at  
505 nm of light. This absorbance is referred to as  
30 Absorbance A.

      Absorbance B was measured by repeating the  
procedure as shown above but using 0.05 ml of an aqueous  
solution containing 10 uM p-aminobenzoic acid instead of  
0.05 ml of serum, and Absorbance C was measured by  
35 repeating the procedure as shown above but using 0.05 ml  
of distilled water instead of 0.05 ml of serum.

      The activity value (mU/ml) of CP-A can be

1 calculated by the following equation:

$$\text{mU/ml} = \frac{A - C}{B - C} \times 10$$

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By repeating the above-described test 20 times,  
the following results were obtained:

Activity value (mU/ml) = 1.7 (nmol/ml/min)

Coefficient of variation (CV) = 5.3%.

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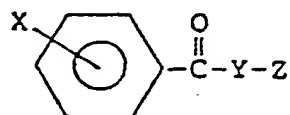
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1 Claims:

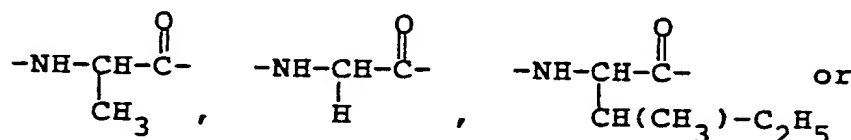
1. A dipeptide derivative having the following general formula:

5

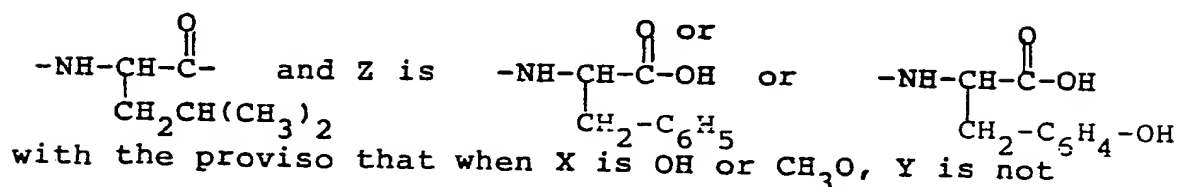


wherein X is OH in the ortho or para position, or NH<sub>2</sub> or CH<sub>3</sub>O in para position, Y is

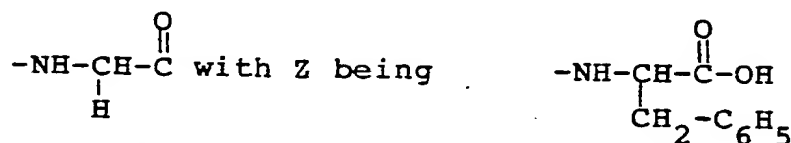
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2. p-hydroxybenzoyl-L-alanyl-L-phenylalanine.

3. p-hydroxybenzoylglycyl-L-tyrosine.

4. o-hydroxybenzoyl-L-isoleucyl-L-phenylalanine.

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5. p-methoxybenzoyl-L-leucyl-L-phenylalanine.

6. p-aminobenzoyl-L-alanyl-L-tyrosine.

7. A method of determining the activity of carboxypeptidase A comprising the steps of

(1) bringing into contact with each other in a liquid medium containing the enzyme carboxypeptidase A, (i), as substrate, a dipeptide derivative as defined in claim 1, (ii) hippuricase, (iii) 4-aminoantipyrine and (iv) an oxidising agent capable as such of oxidising X-benzoic acid, obtainable from the dipeptide and produced in situ to yield a substance which reacts with 4-aminoantipyrine present to yield a quinonimine dye;

1           (2) colourimetrically measuring the concentration  
of the quinonimine dye and

          (3) calculating the activity of said enzyme from  
the concentration of the quinonimine dye.

5           8. A method as claimed in claim 7, wherein said  
substrate is selected from p-hydroxybenzoyl-L-alanyl-  
L-phenylalanine, p-hydroxybenzoylglycyl-L-tyrosine and  
o-hydroxybenzoyl-L-isoleucyl-L-phenylalanine.

10          9. A method of determining the activity of  
carboxypeptidase A comprising the steps of:

          (1) bringing into contact with each other in a  
liquid medium containing the enzyme carboxypeptidase A,  
(i), as substrate, a dipeptide derivative as defined in  
claim 1 (ii) hippuricase, (iii) NADPH or NADH and (ii) a  
15 benzoate hydroxylase having an identity corresponding to  
the meaning and position of X in the general formula of  
the dipeptide derivative;

          (2) spectrophotometrically measuring the decrease  
in absorbance by NADPH or NADH at about 340 nm of light;

20          (3) calculating the activity of said enzyme from  
the decrease in absorbance by NADPH or NADH.

          10. A method as claimed in claim 9, wherein the  
substrate (i) is p-hydroxybenzoylglycyl-L-tyrosine,  
o-hydroxybenzoyl-L-isoleucyl-L-phenylalanine or  
25 p-methoxybenzoyl-L-leucyl-L-phenylalanine and the  
benzoate hydroxylase (ii) is p-hydroxybenzoate  
hydroxylase, salicylate hydroxylase or p-methoxybenzoate  
hydroxylase respectively.  
respectively.

30          11. A method of determining the activity of  
carboxypeptidase A comprising the steps of:

          (1) bringing into contact with each other in a  
liquid medium containing the enzyme carboxypeptidase A,  
(i), as substrate, a dipeptide derivative as claimed in  
35 claim 1, wherein X is essentially  $-NH_2$  and (ii)  
hippuricase;

          (2) adding  $NaNO_2$  to the reaction mixture to

1 produce a diazonium compound;

(3) adding a coupling agent to produce an azo dye;

(4) colourimetrically measuring the concentration  
5 of the azo dye; and

(5) calculating the activity of said enzyme from the concentration of the azo dye.

12. A method as claimed in claim 11, wherein the substrate (i) is p-aminobenzoyl-L-alanyl-L-tyrosine.

10 13. A diagnostic composition for use in determining the activity of carboxypeptidase A which comprises a dipeptide compound as defined in Claim 1 and hippuricase.

14. A diagnostic composition as claimed in claim  
15 13, which additionally contains 4-aminoantipyrine and an oxidising agent capable as such of oxidising X-benzoic acid, wherein X has the meaning given in claim 1, in situ in the composition to yield a substance which reacts with 4-aminoantipyrine to yield a quinonimine dye.

20 15. A diagnostic composition as claimed in claim 13, which additionally contains NADPH or NADH and a benzoate hydroxylase having an identity corresponding to the meaning and position of X in the dipeptide compound of the composition.

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FIG. 1

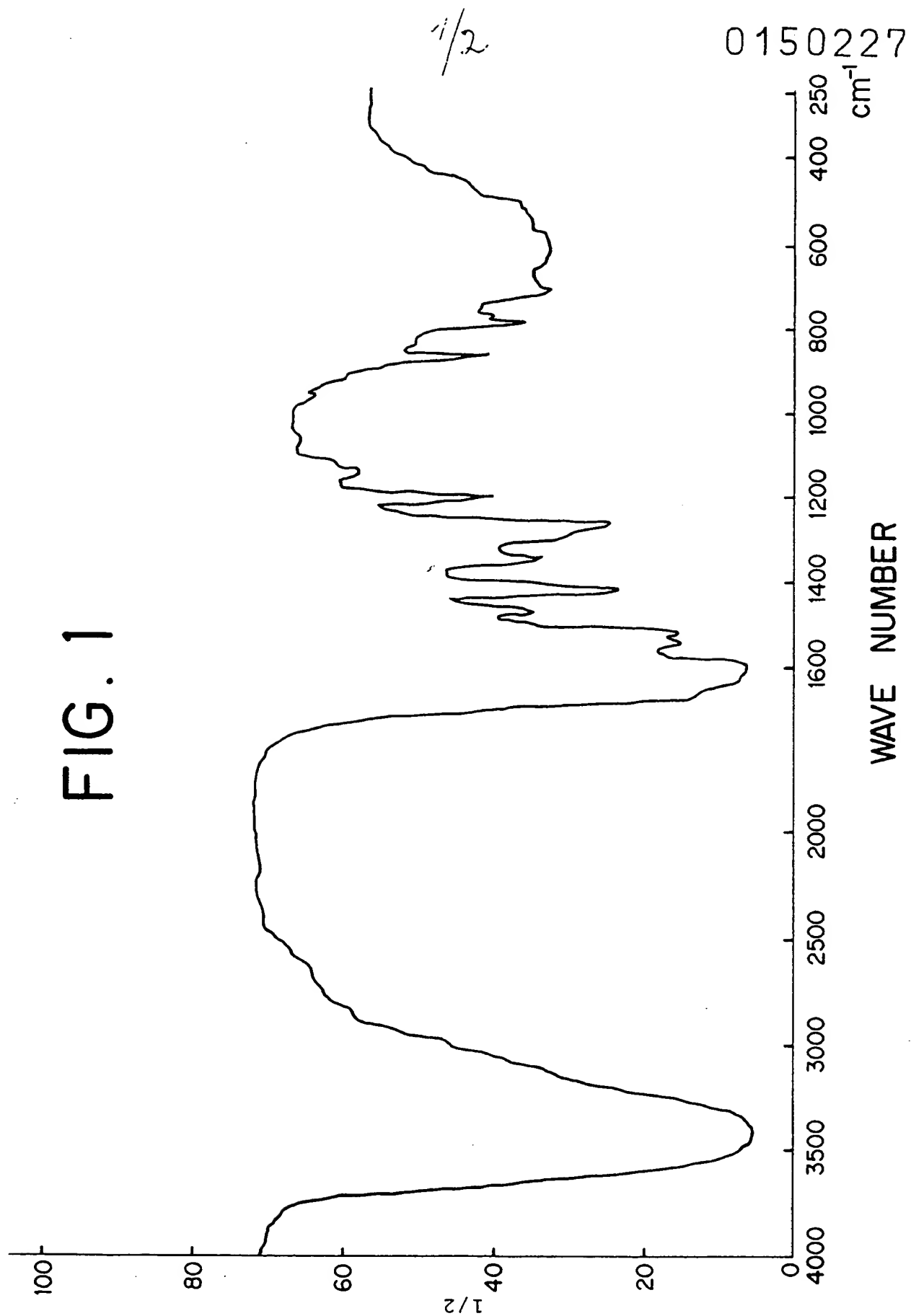
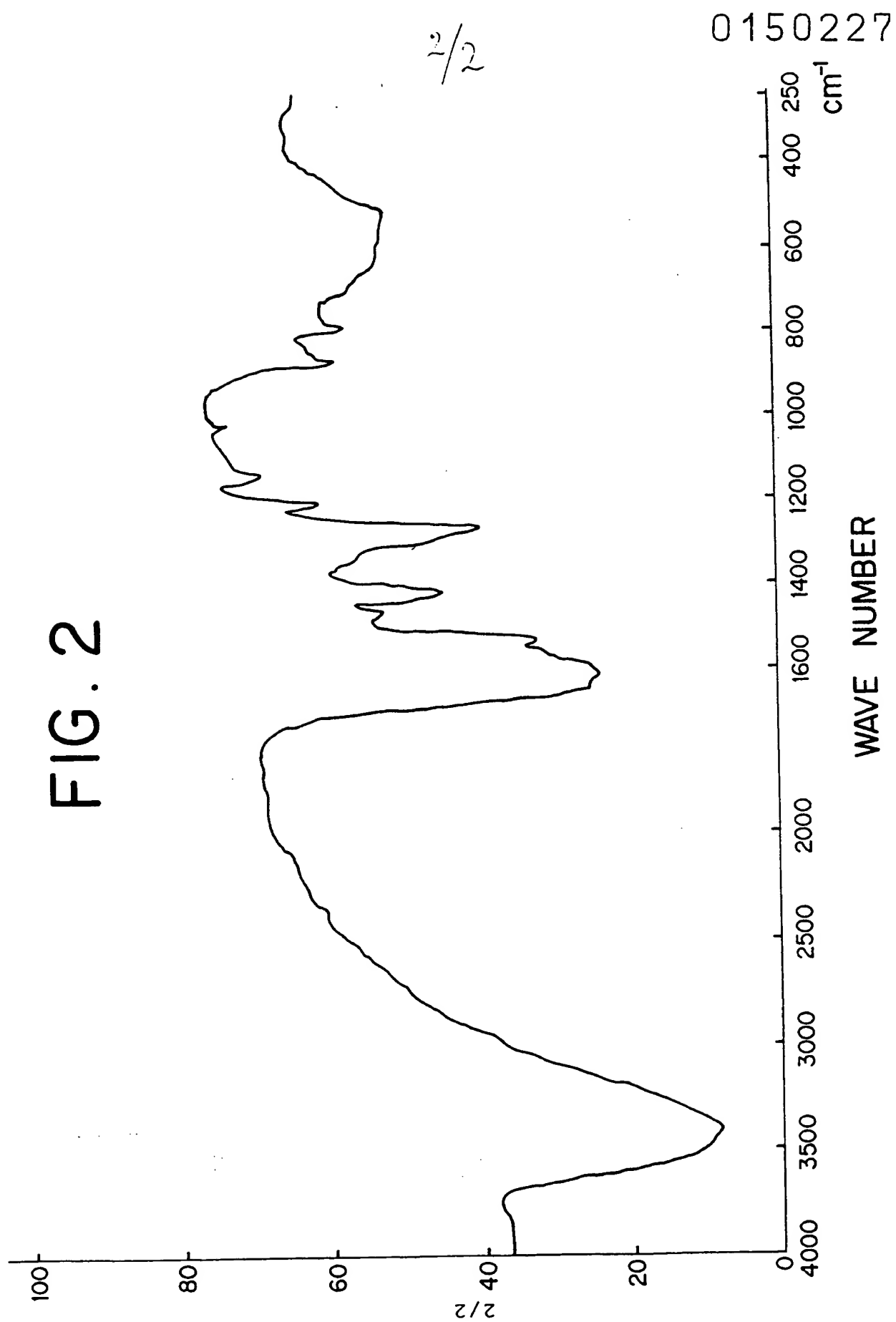


FIG. 2





DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 7)
D, Y	EP-A-0 083 857 (FUJIZOKI PHARM.) * Whole document *	1-15	C 07 C 103/52 C 12 Q 1/36
Y	--- CHEMICAL ABSTRACTS, vol. 95, no. 7, 17th August 1981, page 766, no. 62664q, Columbus, Ohio, US; A.M. EL-NAGGAR et al.: "Synthesis of some 2-hydroxybenzoyl di- and tripeptide derivatives" & EGYPT. J. CHEM. 1979 (Pub. 1980). 22(5), 397-401 * Abstract *	1-15	
Y	--- ARCHIV DER PHARMAZIE, vol. 299, no. 2, February 1966, pages 179-188, Weinheim DE; K.-H. FRÖMMING et al.: "Die Darstellung einiger peptidartiger Derivate der Salicylsäure" * Whole article, particularly pag 181, example XXIV *	1-15	TECHNICAL FIELDS SEARCHED (Int. Cl. 7)  C 07 C 103/00
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 30-08-1984	Examiner ALLARD M.S.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons  & : member of the same patent family, corresponding document	